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Preoperative Assessment of Tumor Angiogenesis by Vascular Endothelial Growth Factor mRNA Expression in Homogenate Samples of Breast Carcinoma: Fine-Needle Aspirates vs. Resection Samples

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Background and Objectives: Tumor angiogenesis is receiving increased attention as a prognostic factor and also as a possible target for new anticancer agents. We investigated whether extent of vascular endothelial growth factor (VEGF) mRNA expression correlated with degree of neovascularization, and whether this expression in fine-needle aspirates could be a marker for assessing angiogenic potential of breast tumors.

Methods: VEGF mRNA expression was semiquantitated by reverse transcriptase-polymerase chain reaction (RT-PCR) followed by Southern blotting. Tumor neovascularization was assessed by immunohistochemical staining with anti-CD31 (PECAM) antibody.

Results: There was a positive correlation between degree of neovascularization and semiquantitated VEGF mRNA expression in invasive ductal carcinomas ($r^2 = 0.346$, $n = 48$, $P < 0.05$). Extent of VEGF mRNA expression in fine-needle aspirates was closely correlated with that in resected invasive ductal carcinomas equal to or less than 3 cm in size ($r^2 = 0.874$, $n = 14$, $P < 0.05$).

Conclusion: These data suggest that semiquantitation of VEGF mRNA expression in fine-needle aspirates is useful for assessing angiogenic potential of invasive ductal carcinomas.

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KEY WORDS: **diagnosis; predictive factor; chemotherapy**

INTRODUCTION

The degree of tumor neovascularization is thought to be of prognostic value in breast carcinomas [1,2]. Among the angiogenic cytokines regulating tumor angiogenesis, vascular endothelial growth factor (VEGF) has been suggested to be responsible for breast tumor angiogenesis [3–5]. In several studies, it has been shown that expression of VEGF confers growth advantage to solid tumors and does promote distant organ metastasis [6,7]. It has been reported also that activation of *ras* oncogenes, the most common oncogenes in human tumors, up-regulates VEGF expression, whereas wild-type p53 tumor suppressor genes down-regulate the expression [8,9]. Thus the angiogenic phenotype is controlled by the balance of activation of oncogenes and inactivation of tumor suppressor genes.

Tumor angiogenesis, moreover, has been focused as a

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TABLE I. Clinical Characteristics of 54 Invasive Breast Carcinomas

Histologic type	Number	Tumor size[a]	LN meta[b]	Distant meta[c]
Invasive ductal (ID) carcinoma	48	2.5 ± 1.4	21	5
Mucinous carcinoma	3	2.7 ± 0.3	0	0
Invasive lobular (IL) carcinoma	1	6.0	1	1
Mixed type of ID and IL[d]	1	2.0	1	0
Medullary carcinoma	1	2.6	0	0

[a]Tumor size was expressed as mean ± S.D.
[b]Number of patients with lymph node (LN) metastasis.
[c]Number of patients with distant organ metastasis.
[d]Mixed invasive ductal and lobular carcinomas.

target of new anticancer drugs, i.e., angiogenesis inhibitors [10,11]. In fact, secretion of angiogenic factors by several tumors has been reported to induce angiogenesis, leading to the suggestion that inhibition of angiogenesis may present a potent and selective therapeutic approach for a large variety of metastatic cancers [12].

In this study, we determined the differential mRNA expression in VEGF in breast carcinomas by reverse transcriptase-polymerase chain reaction (RT-PCR) followed by Southern blot and evaluated the possible assessment of tumor neovascularization by the extent of VEGF mRNA expression in fine-needle aspirates. Diagnosis of breast carcinoma by fine-needle aspiration cytology is increasingly common [13]. With the application of RT-PCR and Southern blot to fine-needle aspirates, a subset of tumors could be distinguished based on angiogenic potential before therapeutic approach.

MATERIALS AND METHODS

Sample Collection

Breast cancer specimens from 54 patients were assessed (Table 1). All tumors were primary invasive carcinomas. None of the patients received preoperative radiotherapy or chemotherapy. Tumor samples were collected in the operating room and processed within 30 minutes. Each sample was divided into two blocks. One was minced in RNA lysis buffer on ice for extraction and subsequent analysis by RT-PCR Southern blot. The other block was fixed in 10% formalin for quantification of vascularity by immunohistologic staining.

Fine-needle aspiration biopsy of 21 carcinomas was performed at the same time as a routine procedure for cytologic diagnosis, just before a radical operation in the operating room with the patient under anesthesia, or immediately after the tumor specimen was resected. Samples were taken using 22 or 23 gauge needles from the center of the tumor and immediately put into RNA lysis buffer. The 21 tumors were all invasive ductal carcinomas by histologic examination of the resected specimens.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

PCR oligonucleotide primers for VEGF and β-actin were designed to bracket cDNA sequences that in genomic DNA cross an intron-exon boundary [14,15]. Primer sequences were as follows: VEGF, 5′: ATGAACTTTCTGCTGTCTTGG, 3′: TCACCGCCTCGGCTTGTCACA; β-actin, 5′: GTGGGGCGCCCCAGGCACCA, 3′: CTCCTTAATGTCACGCACGATTTC. The primers for VEGF were able to detect two of four different molecular species produced by alternative splicing of mRNA-VEGF165 and VEGF121. The predicted size for VEGF was 576 bp and 444 bp, and for β-actin was 541 bp.

Total RNA of primary tissues and fine-needle aspirates was isolated by single step, guanidinium thiocyanate-phenol-chloroform extraction [16]. RT-PCR was carried out according to the Perkin Elmer Cetus protocol with some modifications as described previously [5]. Each RT reaction was carried out with 1.0 μg of total RNA per sample. The 20 μl solutions of cDNA obtained were diluted two times with diethyl pyrocarbonate (DEPC) water. The cDNA amplification for VEGF was performed with 2 μl of the diluted solution at 92°C for denaturation, 52°C for annealing, and 72°C for extension. Each step was 1 minute in duration. Ten cycles were preformed. This amplification was followed by 20 cycles with an annealing temperature of 55°C. cDNA amplification for β-actin was performed for 30 cycles with annealing temperatures of 58°C.

Validation of PCR Cycles for Semiquantitative Assay

To validate our PCR cycles for VEGF (described above), we quantitated the PCR products by Southern blot (described below) with serial dilutions of cDNA. Starting with cDNA in the 2 μl diluted solution, which was originally 50 ng of total RNA, 10-fold serial dilutions were performed for the sample expressing the most dense VEGF band. There was a linear correlation be-

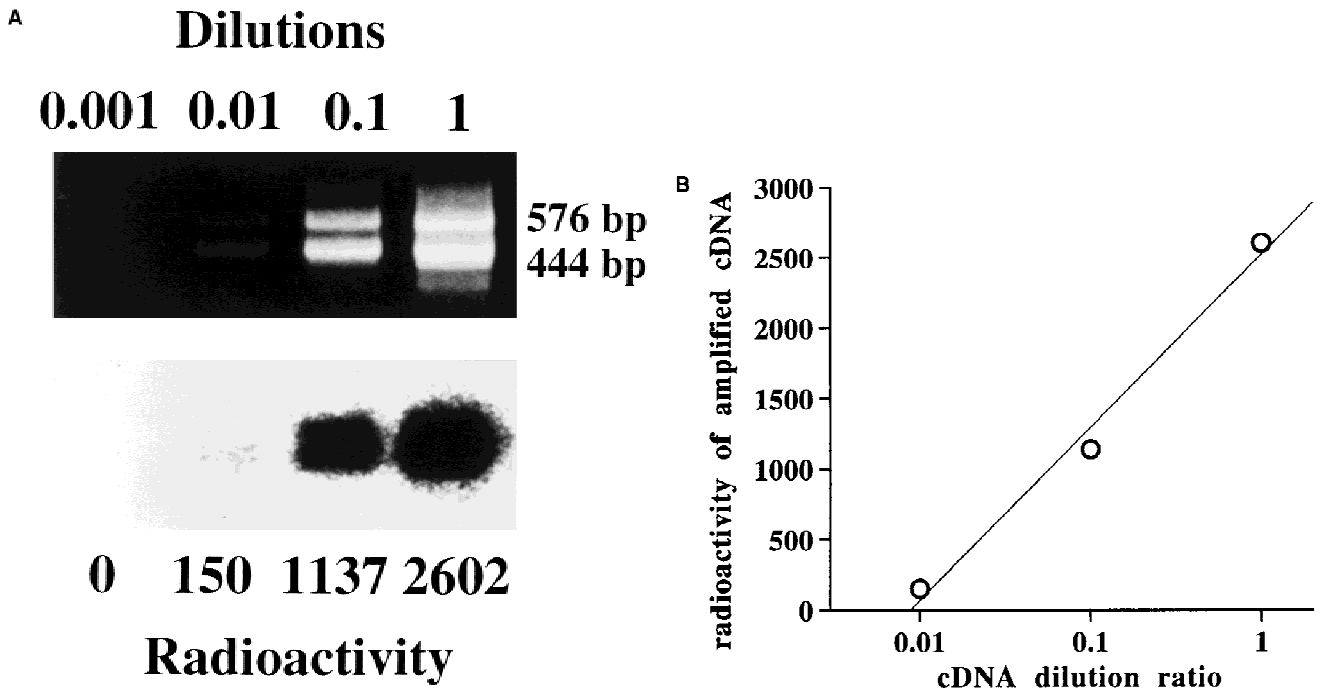

Fig. 1. Southern blot analysis for variation of semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) for vascular endothelial growth factor (VEGF) mRNA expression. Starting with cDNA in 2 μl diluted solution, which was originally 50 ng of total RNA, 10-fold serial dilutions were performed for the sample expressing the most dense VEGF band. The cDNA amplification was performed with 2 μl of the diluted solutions at 92°C for denaturation, 52°C for annealing, and 72°C for extension. Each step was 1 minute in duration. Ten cycles were performed. This amplification was followed by 20 cycles with an annealing temperature of 55°C. This was optimum cycle for semiquantitation of VEGF mRNA expression **(A).** There was a linear correlation between logarithms of dilution and expression amounts **(B).**

tween logarithms of dilution and expression amounts (Fig. 1).

Southern Blot

After the electrophoresis of PCR products from resected tumor samples and fine-needle aspirates, Southern transfer onto a nylon membrane (Bio-Rad, Hercules, CA) was performed. Hybridization was carried out at 58°C with a solution of 6X SSC/5X Denhardt/0.1% sodium dodecylsulfate (SDS) using [γ-^{32}P]ATP labeled 20-mer oligonucleotide probes corresponding to the sequences between the primers for VEGF and β-actin, respectively. The sequences for each probe were as follows: VEGF, 5′: GATGTTGGACTCCTCAGTGG; β-actin, 5′: CTGCTGACCGAGGCCCCCCT. After overnight hybridization, the nylon membranes were washed twice in 2X SSC/1% SDS for 5 minutes, twice in 0.16X SSC/1% SDS for 15 minutes, and once in 2X SSC/1% SDS for 5 minutes. Radioactivity was determined using a BAS 2000 Bioimage Analyzer (Fujix, Tokyo, Japan). In order to ascertain the minimum variance between the nylon membranes in Southern blot procedure, PCR products of VEGF and β-actin from a human breast cancer cell line Br.M (originating from a primary ductal carcinoma) were also electrophoresed and transferred as a control. Each quantity of VEGF mRNA expression was corrected by that of corresponding β-actin mRNA expression.

Assessment of Vascularization

Blood vessels were visualized by staining endothelial cells with JC70 (Dako Japan, Kyoto, Japan) [17], a monoclonal antibody to CD31, which is known as endothelial cell adhesion molecule or platelet/endothelial cell adhesion molecule. A standard immunoperoxidase technique was utilized [18]. Vascular counting was performed according to the method of Weidner et al. [1] with some modifications. Areas representative of the invasive component of the cancer were selected from sections stained with hematoxylin and eosin. The most vascularized area of the tumor was located at low magnification, and the vessels were counted on three 200X fields (0.64 mm^2/field) by two independent observers who were not aware of the experimental protocol. Vascular counts of three different parts of the most vascularized area were recorded. The mean of the three vascular counts was reported as the vascular count of the tumor.

Statistics

The Spearman rank correlation test was used for correlation analysis between the vascular count and VEGF

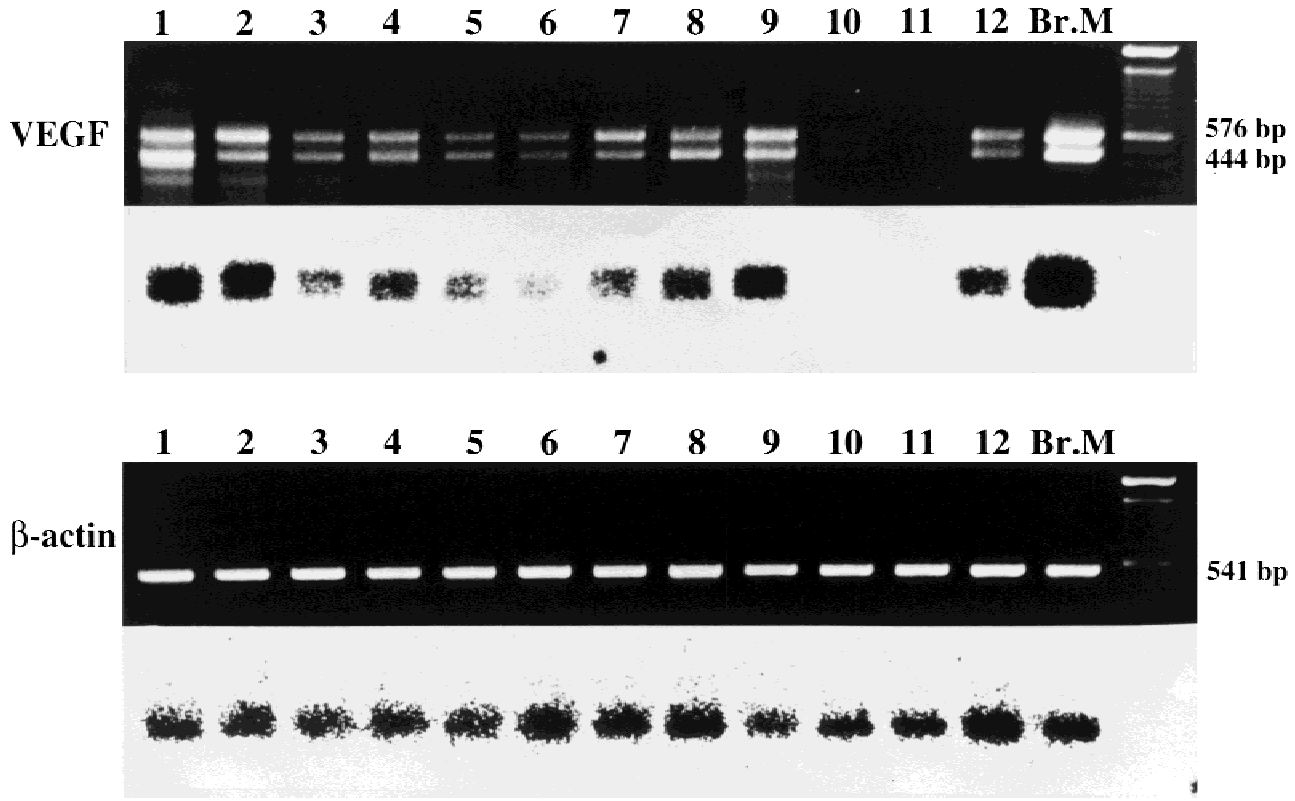

Fig. 2. Representative Southern blot bands for vascular endothelial growth factor (VEGF) mRNA expression in breast carcinomas and for β-actin as an internal control. Bands for VEGF showed various densities (from faint to dense), whereas the bands for β-actin were uniform.

mRNA expression in tumor tissues and fine-needle aspirates. A *P*-value of < 0.05 was considered statistically significant.

RESULTS

Correlation Between Vascular Counts and Semiquantitated VEGF mRNA Expression in Tumor Tissues

The mean vascular count of all of the tumor tissues was 74.7/200X field (median: 72, range: 23–197, n = 54). The mean vascular count of the invasive ductal carcinomas was 77.5/200X field (median: 72.5, range: 32–197, n = 48).

All samples showed dense bands at 541 bp (β-actin) on agarose gels. Clear positive bands for VEGF at predicted size were apparent in 46 out of 54 (85.2%) samples. The PCR products were transferred onto nylon membranes and were semiquantitated with radiolabeled specific oligo probes. All PCR products for VEGF showed positive radioactivity. The bands for VEGF exhibited various densities, (from faint to dense), while the bands for β-actin were similar (Fig. 2). Overall, there was a positive correlation between vascular counts and semiquantitated VEGF mRNA expression in the resected tumor tissues ($r^2 = 0.255$, n = 54, $P < 0.05$). In the invasive ductal carcinomas, there was an intense correlation between vascular counts and semiquantitated VEGF mRNA expression ($r^2 = 0.346$, n = 48, $P < 0.05$, Fig. 3). Although the numbers of tumors were low in the other histologic tumor types, no correlation was observed between these variable (Fig. 4). The mucinous carcinomas expressed moderate amounts of VEGF mRNA but showed low tumor vascularity. One invasive lobular carcinoma expressed an extremely low amount of VEGF mRNA.

Comparison of VEGF mRNA Expression in Fine-Needle Aspirates and Resected Tumor Specimens

Fine-needle aspiration biopsy was performed 29 times in 21 tumors. Subsequent RNA extraction resulted in 26 samples from 20 tumors. The mean amount of total RNA extract from fine-needle aspirates was 5.2 μg (1 to 30 μg), which was enough to be used for RT-PCR analysis. After Southern blotting for VEGF and β-actin, the amount of VEGF mRNA expression in fine-needle aspirates was calculated. Overall, there was a significant correlation between extent of VEGF mRNA expression in fine-needle aspirates and that in resected tumor specimens ($r^2 = 0.653$, n = 20, $P < 0.05$). When large tumors (>3 cm in diameter) were excluded, there was an intense correlation between extent of VEGF mRNA expression

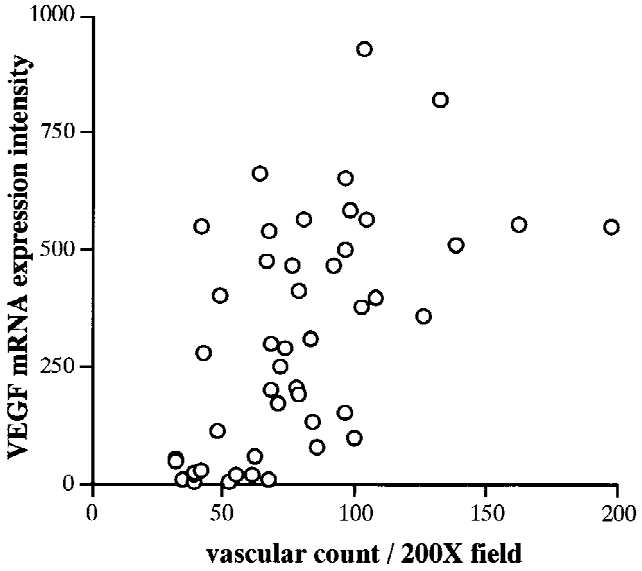

Fig. 3. Correlation between vascular counts and semiquantitated vascular endothelial growth factor (VEGF) mRNA expression in invasive ductal carcinomas ($r^2 = 0.346$, n = 48, $P < 0.05$).

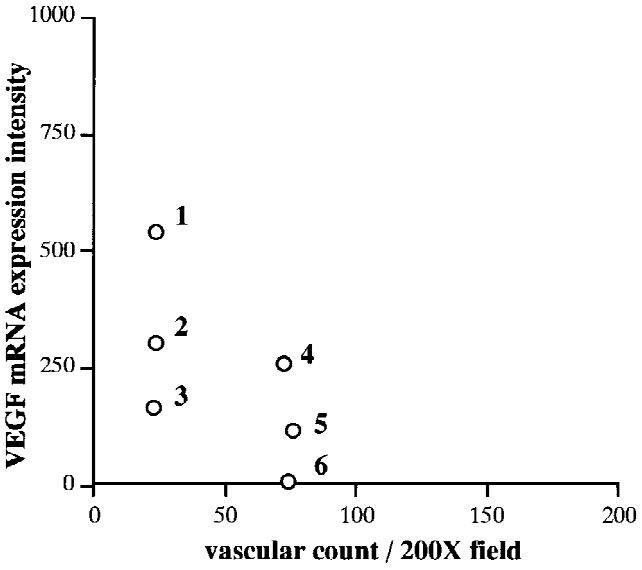

Fig. 4. Vascular counts and semiquantitated vascular endothelial growth factor (VEGF) mRNA expression in breast carcinomas other than invasive ductal carcinoma. 1–3, mucinous carcinoma; 4, medullary carcinoma; 5, mixed invasive ductal and lobular carcinomas; 6, invasive lobular carcinoma.

in fine-needle aspirates and that in resected tumor specimens ($r^2 = 0.874$, n = 14, $P < 0.05$) (Figs. 5, 6).

DISCUSSION

In the present study, we showed that the angiogenic phenotype of invasive ductal carcinomas of the breast can be assessed by quantitation of VEGF expression and showed the possible diagnostic value of VEGF mRNA expression in fine-needle aspirates from primary breast

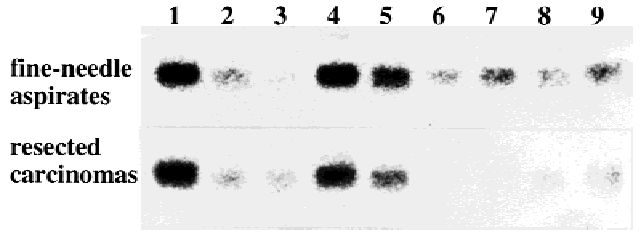

Fig. 5. Representative Southern blot bands for vascular endothelial growth factor (VEGF) mRNA expression in fine-needle aspirates and resected carcinomas.

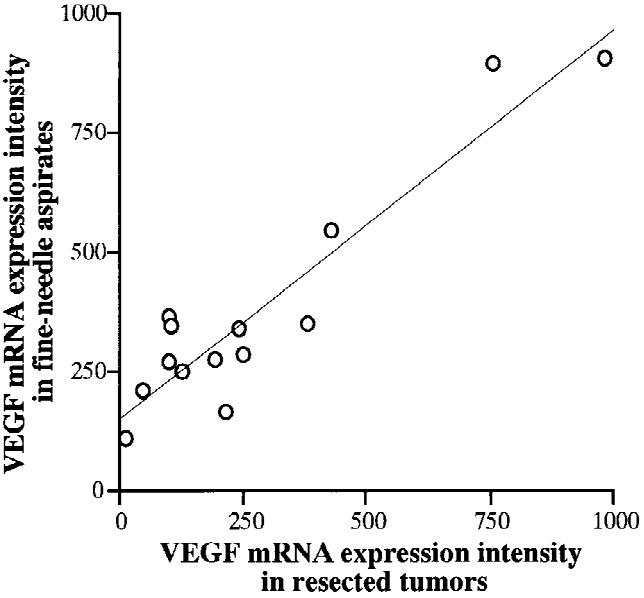

Fig. 6. Correlation between semi-quantitated vascular endothelial growth factor (VEGF) mRNA expression in fine-needle aspirates and that in resected tumor specimens equal to or less than 3 cm in size. There was statistically significant correlation between these samples ($r^2 = 0.874$, n = 14, $P < 0.05$).

carcinomas. There was a positive correlation between the amount of VEGF mRNA expression and tumor vascularity in invasive ductal carcinomas using semiquantitative RT-PCR followed by Southern blotting. This finding is consistent with several previous studies that showed increased expression of VEGF in invasive ductal carcinoma of the breast and its correlation with increased vascularity of the tumors [3–5,19].

Not only confirming the previous studies, the present study also offers a significant new insight that quantitating the amount of VEGF mRNA expression in fine-needle aspirates may be useful for assessing the angiogenic potential of a breast tumor before therapeutic approach. The total RNA extracted from fine-needle aspirates was of sufficient quantity to be used for RT-PCR analysis. A statistically significant correlation was observed between extent of VEGF mRNA expression in fine-needle aspirates and that in resected tumor specimens. As we expected, when large tumors (>3 cm in

diameter) were excluded, there was an intense correlation of the extent of VEGF mRNA expression between these samples. It has been suspected that the lack of correlation in the large tumors was due to greater intratumor regional differences in VEGF mRNA expression compared to the smaller carcinomas. In fact, in a preliminary study, we observed greater intratumor regional differences in VEGF mRNA expression in large lesions than in smaller ones (data not shown). Smaller carcinomas with less intratumor regional difference, i.e., <3 cm in size, would be appropriate targets for this new diagnostic approach.

Current therapeutic strategies for individual patients with breast carcinoma frequently are determined by axillary lymph node status, tumor size, estrogen status, and the pathologic stage of disease at diagnosis. However, it is noteworthy that tumors recur in some patients with small carcinomas free of axillary lymph node metastasis on conventional histologic examination. To obtain a more profound understanding of the molecular basis of breast carcinomas leading to better survival of patients, many have investigated the prognostic or predictive value of various oncogenes and growth factors related to tumor angiogenesis, invasion, or metastasis [20,21]. However, only a few have done with preoperatively obtained fine-needle aspirates [22,23]. The present data suggest that semiquantitation of VEGF mRNA expression in fine-needle aspirates is useful for assessing the biologic potential of angiogenesis and metastasis of invasive ductal carcinomas <3 cm in size.

In the histologic types of breast tumors other than invasive ductal carcinoma (mucinous carcinoma, invasive lobular carcinoma, and medullary carcinoma), no correlation was observed between the amount of VEGF mRNA expression and tumor vascularity, although the numbers of tumors for these other histological types were low. Mucinous carcinomas showed low tumor vascularity but expressed moderate amounts of VEGF mRNA. Brown et al. [4] have reported in an in situ hybridization study that VEGF mRNA was expressed at low levels in invasive lobular carcinoma and at high levels in invasive ductal carcinoma. VEGF expression and tumor vascularity may differ with the histologic tumor type. Further studies should be performed to confirm this speculation.

In conclusion, semiquantitation of VEGF mRNA expression in fine-needle aspirates is useful for assessing angiogenic potential of a subset of breast carcinomas, which may provide a guide for treatment.

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